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Regulation and Properties of PstSCAB, a High-Affinity, High-Velocity Phosphate Transport System of *Sinorhizobium meliloti*

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The properties and regulation of the *pstSCAB*-encoded P_i uptake system from the alfalfa symbiont *Sinorhizobium meliloti* are reported. We present evidence that the *pstSCAB* genes and the regulatory *phoUB* genes are transcribed from a single promoter that contains two PhoB binding sites and that transcription requires PhoB. *S. meliloti* strain 1021 (Rm1021) and its derivatives were found to carry a C deletion frameshift mutation in the *pstC* gene (designated *pstC1021*) that severely impairs activity of the PstSCAB P_i transport system. This mutation is absent in RCR2011, the parent of Rm1021. Correction of the *pstC1021* mutation in Rm1021 by site-directed mutagenesis revealed that PstSCAB is a P_i-specific, high-affinity (K_m, 0.2 μM), high-velocity (V_{max}, 70 nmol/min/mg protein) transport system. The *pstC1021* allele was shown to generate a partial *pho* regulon constitutive phenotype, in which transcription is activated by PhoB even under P_i-excess conditions that render PhoB inactive in a wild-type background. The previously reported symbiotic Fix$^-$ phenotype of *phoCDET* mutants was found to be dependent on the *pstC1021* mutation, as Rm1021 *phoCDET* mutants formed small white nodules on alfalfa that failed to reduce N_2, whereas *phoCDET* mutant strains with a corrected *pstC* allele (RmP110) formed pink nodules on alfalfa that fixed N_2 like the wild type. Alfalfa root nodules formed by the wild-type RCR2011 strain expressed the low-affinity *orfA-pit*-encoded P_i uptake system and neither the *pstSCAB* genes nor the *phoCDET* genes. Thus, metabolism of alfalfa nodule bacteroids is not P_i limited.

The transport of inorganic phosphate or other sources of phosphorus is essential for growth of all living organisms. In most soil environments, the concentration of soluble or biologically available phosphate is in the micromolar range, and it seems likely that many soil microorganisms live under P_i-limiting growth conditions (8, 10). It is known that many microorganisms have the ability to change their metabolism in response to the amount of phosphorus available for cellular growth. The switch in metabolism is mediated through the repression and induction of transcription of various genes whose products are involved in processes ranging from the uptake and acquisition of sources of phosphorus to the de novo synthesis of new cellular components that allows redistribution of, or replacement of, molecules such as phospholipids that represent large reservoirs of phosphorus within the cell (4, 5, 27, 29, 61, 62, 63, 67). In many gram-negative bacteria, *phoB* regulates expression of genes whose expression responds to exogenous phosphorus concentrations. The response regulator PhoB, together with its cognate sensor histidine kinase, PhoR, has been well studied in *Escherichia coli*, and genes whose expression is regulated by the PhoB protein are referred to as members of the Pho regulon (11, 47, 71).

Sinorhizobium meliloti is a gram-negative α-proteobacterium that forms N_2-fixing root nodules on alfalfa. Our analysis of the P_i transport systems of *S. meliloti* resulted from identification of a locus on the pSymB megaplasmid that was required for the development of wild-type N_2-fixing nodules (Fix$^+$) (16, 17). This locus comprised the *phoCDET* genes, which encode an ABC-type high-affinity transport system, and we demonstrated that this system could transport P_i and likely phosphonates (4, 69). *S. meliloti* strains carrying mutations in the *phoCDET* genes formed small white nodules on alfalfa that contained few bacteroids and failed to fix N_2 (Fix$^-$) (4, 16). In further studies, we identified two classes of second-site mutations that suppressed the Fix$^-$ phenotype of *phoCDET* mutants to Fix$^+$. Genetic and biochemical analyses revealed that one of the suppressor mutant classes carried mutations that were located in the promoter and increased transcription of the *orfA-pit* genes, which encode a low-affinity P_i transport system (6, 48, 69). These mutations are close to a putative PhoB binding site in the *orfA-pit* operon. The other suppressor mutations mapped to the *phoB* locus, and *phoB* null alleles were found to suppress the *phoCDET* Fix$^-$ phenotype. PhoB was subsequently shown to be a positive regulator of *phoCDET* transcription and a negative regulator of *orfA-pit* transcription (5). All of the data suggested the following model for *orfA-pit* and *phoCDET* regulation. In *S. meliloti* cells growing in the presence of excess P_i (2 mM), the *orfA-pit* genes are expressed, and P_i is transported via the low-affinity OrfA-Pit transport system. Under these P_i-excess conditions, PhoB is inactive, and the *phoCDET* genes are not expressed. Under P_i-limiting conditions, the *orfA-pit* genes are repressed by activated PhoB, the *phoCDET* genes are expressed, and P_i is transported via the high-affinity PhoCDET system.

Our examination of the P_i transport systems of *S. meliloti* Rm1021 suggested that only two transport systems, PhoCDET and OrfA-Pit, were functional. However, sequence analysis of the region upstream of the *S. meliloti phoUB* genes revealed the presence of genes homologous to the *p*hosphate-*s*pecific *t*ransport genes, *pstSCAB*, that have been best characterized in *E. coli* (58, 64, 74). Many bacterial strains contain products of

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pstSCAB homologs that function as high-affinity phosphate transporters (13, 31, 32, 45, 46, 53). The PstSCAB proteins comprise an ABC-type transport system in which PstS is a periplasmic P_i binding protein, PstC and PstA are integral membrane proteins, and PstB is the ATP binding protein (15, 37, 65).

Here we describe genetic and biochemical studies that were performed to determine the role of the *pstSCAB* gene cluster in gene regulation and P_i assimilation in *S. meliloti*. We found that the *pstC* gene in *S. meliloti* strain 1021 carries a frameshift C deletion mutation, designated *pstC1021*, that can be corrected by insertion of a cytosine at the original mutant site or through addition of a guanine base 42 nucleotides upstream of the original C deletion. Regulatory and phenotypic effects of the *pstC1021* mutation were investigated, and the previously reported Fix^- phenotype of *phoCDET* mutants was shown to be dependent on the *pstC1021* allele found in all Rm1021-derived strains. The biochemical properties of the wild-type PstSCAB transport system were determined, and the implications of the new data for the previous analysis of the *phoCDET* and *orfA-pit* genes of *S. meliloti* are discussed below.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The strains and plasmids employed in this work are listed in Table 1. The *E. coli* strains were grown in Luria-Bertani (LB) medium, and the *S. meliloti* strains were grown in LB medium containing 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LBmc). The phosphate-free medium was morpholinopropanesulfonic acid (MOPS)-buffered minimal medium (4) containing 0.3 μg/ml biotin and 10 ng/ml $CoCl_2$ (72) supplemented with an appropriate filter-sterilized carbon source. For growth experiments we used the protocol described by Bardin and Finan (5). *S. meliloti* cultures were grown overnight in LBmc and washed with MOPS medium with no phosphate added (MOPS P0); 20 μl of the cells was used to inoculate 5 ml of MOPS P0 (optical density at 600 nm [OD_{600}], ~0.05). The cells were grown for 24 h with agitation, and the OD_{600} of the resulting cultures were adjusted to 0.2; 200-μl portions of these cultures were then used to inoculate 20 ml of MOPS medium supplemented with 2 mM orthophosphate (MOPS P2) or MOPS P0 in disposable tissue culture bottles.

When necessary, media were supplemented with the following antibiotics: ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), gentamicin (8 μg/ml for *E. coli* and 30 μg/ml for *S. meliloti*), kanamycin (20 μg/ml for *E. coli*), neomycin (200 μg/ml for *S. meliloti*), streptomycin (200 μg/ml for *S. meliloti*), and tetracycline (10 μg/ml for *S. meliloti* and 7 μg/ml for *E. coli*).

DNA manipulation and genetic techniques. Cloning procedures, including DNA isolation, restriction digestion, ligation, and transformation, were performed as described by Sambrook et al. (59). Conjugal mating with MT616 as the helper strain was performed as previously described (18). ϕM12 generalized transduction was carried out as described by Finan et al. (25).

To isolate the 7.5-kb HindIII DNA fragment containing *S. meliloti phoR-pstSCAB* and a partial *phoU* region, we first isolated an R-prime plasmid carrying this region by using the protocol described by Osteras et al. (49). Construction of R-prime plasmids is based on the ability of plasmid $R_{68.45}$ to mobilize the DNA of the host strain at a high frequency (57). The $R_{68.45}$ derivative pJB3JI (Tc^r) was transferred into RmH615 (*phoB3*::Tn*V*), and the resulting strain was used as the donor in conjugational mating with rifampin-resistant (Rif^r) *E. coli* recipient strain MT620. Transconjugants were selected on LB medium with rifampin (20 μg/ml) and kanamycin (20 μg/ml). Plasmid DNA was isolated from these transconjugants, and the transconjugants carrying R-prime plasmids with genomic DNA contiguous with the *phoB3*::Tn*V* insertion in RmH615 were identified with reference to the DNA sequence of Tn*V* and the *phoR-pstSCAB-phoU* region. The 7.5-kb HindIII gene fragment from one R-prime plasmid was subcloned into pUC119 to obtain pTH691, and the border sequence of this fragment was confirmed by sequencing using the −48 universal primer.

To make *lacZ-aacC1* cassette (9) or ΩSp cassette (52) insertions in the *S. meliloti pstB* gene, the 2.7-kb HindIII-EcoRI DNA fragment containing the *pstAB* region was subcloned from pTH691 into the pK18GIImob vector (34) to obtain plasmid pTH659, and the *lacZ-aacC1* cassette was inserted into the SmaI site of the *pstB* gene in two orientations to obtain plasmids designated pTH663 (*lacZ-aacC1* in the orientation opposite that of *pstB*) and pTH664 (*lacZ-aacC1* in same orientation as *pstB*). The ΩSp cassette was inserted into the same SmaI site of pTH659 to obtain plasmid pTH665 (Fig. 1A). *S. meliloti* strains RmK385, RmK386, and RmK390 carrying the mutations described above were identified following transfer of the appropriate Gm^r plasmid into RmG212 and subsequent screening for double-crossover recombinants with subsequent loss of the Nm^r vector marker. This generated strains. RmK426 was constructed by transducing neomycin resistance from RmH615 (p*hoB3*::Tn*V*) into RmK385 and screening for neomycin-resistant transductants.

The *pstS-pstC* intergenic regions from Rm1021, RmG212, RCR2011, Rm5000, RmG830, and the suppressor mutant RmP101 were PCR amplified and sequenced using primers ML3344 (5′-ACGATCAGATGATCGGCCCCGAC-3′) and ML3345 (5′-CCCAGACCGTGCCGAAGAA GAAC-3′), as shown in Fig. 1A. All primers were synthesized at the McMaster University MOBIX facility, and all the sequences were obtained from this facility.

The promoter regions of *pstS*, *orfA-pit*, and *phoC* were PCR amplified and cloned into pTH1582, a modified version of the *parAB gusA* reporter vector pJP2 (51, 77), to generate pTH1734, pTH1735, and pTH1736, respectively. The primers for *pstS* were ML6254 (5′-CGAAGCTTAGCATATCCTCACGCG TCACCG-3′) and ML6255: (5′-AACTCGAGATCAGAGCAGGTTGCCTGC CTC-3′); the primers for *phoC* were ML7605 (5′-CGAAGCTTCTATGCGGTC CAACTCGCTCGC-3′) and ML7606 (5′-AACTCGAGTTGAGCGAGGAGA CCTGTAGGC-3′); and the primers for *orfA-pit* were ML7607 (5′-CGAAGCTTG AGTGCCCGTGCCGATCTCC-3′) and ML7608 (5′-GATTTCGTCGGCCTC ATTCTCGAGCG-3′).

Site-directed mutagenesis. To convert the *pstC1021* mutation to the wild type, the 1.4-kb PstI-EcoRI fragment containing the *pstS-pstC* intergenic region (Fig. 1A) was cloned into the pJQ200-SK vector (54) to obtain plasmid pTH1906. Using pTH1906 as the template, PCR mutagenesis was performed using primers ML3567 (5′-ACCCGATTGCGGGCACGGAAACGGACGTCG-3′) and ML3568 (5′-AC GTCCGTTTCCGTGCCCGCAATCGGGTGG-3′). *Pfu* Turbo DNA polymerase from Stratagene was used in the PCR, and the PCR product was digested with DpnI (New England Biolabs). The sequences of inserts in pTH1906 carrying the *pstC1021* mutation and the corrected plasmid designated pTH1907 carrying the wild-type RCR2011 *pstC* allele were confirmed by DNA sequencing. Plasmid pTH1907 was transferred into RmG212, RmG490, and Rm1021, and single-crossover recombinants were selected on LB medium containing 40 μg/ml gentamicin. Double-crossover integrants were subsequently obtained by growing cells on LBmc with 5% sucrose and screening for gentamicin-sensitive (Gm^s) colonies as described by Quandt and Hynes (54). Strains Rm1021, RmG212, and RmG490 carrying the corrected wild-type *pstC* gene were designated RmP110, RmP111, and RmP371, respectively. In the same way, pTH1096, which contained the *pstC1021* allele, was integrated into Rm5000, and a recombinant in which the wild-type *pstC* gene was replaced by the *pstC1021* allele was designated RmP379. The DNA sequence of the *pstS-C* region in strains RmP110, RmP111, RmP371, and RmP379 was confirmed following PCR amplification and sequencing with primers ML3344 and ML3345 as described above.

Plant growth, alkaline phosphatase, β-galactosidase, and β-glucuronidase assays. Alkaline phosphatase and β-galactosidase activities were measured as described previously (5). β-Glucuronidase assays were performed as described by Reeve et al. (56). Plant growth experiments in a nitrogen-deficient growth medium were performed as described previously (4). To measure β-glucuronidase activity in nodules, plants were harvested 4 weeks following inoculation, and 7 to 10 nodules were put into a 1.5-ml tube containing 750 μl ice-cold MMS buffer (40 mM MOPS, 20 mM KOH, 2 mM $MgSO_4$, 0.3 M sucrose; pH 7.0). The nodules were crushed and then centrifuged for 2 min at 2,000 rpm. Five hundred microliters of the supernatant was transferred to a new tube, and sodium dodecyl sulfate was added to a final concentration 0.01%. Ten microliters was removed to determine the total protein concentration using the Bio-Rad assay. One hundred microliters of the nodule extract was used in the β-galactosidase assay as described previously (56).

For histochemical GusA staining, the protocol described by Boivin et al. (12) was used, with modifications. Fresh nodules were manually sectioned using a razor blade to obtain 0.5- to 1-mm-thick sections. Fifty microliters of GusA staining solution was added to cover the sections. One hundred milliliters of staining solution contained 50 ml of 0.2 M phosphate buffer (pH 7), 43 ml of double-distilled H_2O, 50 μl of Triton X-100, 4 ml of 0.25 M EDTA (pH 7.0), 1.5 ml of 0.1 M $K_3Fe(CN)_6$ instead of 0.5 ml, and 1.5 ml of 0.1 M $K_4Fe(CN)_6$ instead of 0.5 ml. Five milligrams of X-Gluc (5-bromo-4-chloro-3-indolyl-β-glucuronide) was added to 5 ml of the staining solution. After staining for 2 to 3 h at room temperature (a vacuum could have aided in penetration of the X-Gluc solution), the sections were rinsed two or three times in the staining solution without X-Gluc. Photographs were taken using a Leitz Laborlux-12 microscope (magnification, ×40) and a Nikon Dxm1200F digital camera system.

TABLE 1. Bacterial strains, phage, and plasmids

Strain, phage, or plasmid	Relevant characteristics	Source or reference
Sinorhizobium meliloti strains		
RCR2011	Wild type	Lab stock
Rm1021	SU47 *str-21*	43
Rm5000	SU47 *rif-5*	25
RmG212	Rm1021 *lacZ*	30
RmF921	RmG212 *phoC1.7*::Tn*5*	16
RmG490	Rm1021 *phoC490*::ΩSp, Fix$^-$	16
RmG804	Rm1021 *pit310*::Tn*5*	6
RmG830	Rm1021 *phoC490*::ΩSp *pit310*::Tn*5*	6
RmH615	RmG212 *phoB3*::Tn*V*	5
RmH625	Rm1021 *phoB8*::Tn*V phoC490*::ΩSp, Fix$^+$	5
RmK385	RmG212 *pstB*::*lacZ-aacC1*, APase$^+$	This study
RmK386	RmG212 *pstB*::*lacZ-aacC1*, APase$^-$	This study
RmK390	RmG212 *pstB*::ΩSp, APase$^-$	This study
RmK399	RmG212 *pstB*::ΩSp *phoC1.7*::Tn*5*, APase$^-$	This study
RmK426	RmG212 *pstB*::*lacZ-aacC1 phoB3*::Tn*V*	This study
RmK428	RmG212 *pstB*::*lacZ-Gm phoB3*::Tn*V*	This study
RmP101	RmG830 suppression mutant, Fix$^+$	This study
RmP110	Rm1021 with changed wild-type *pstC*	This study
RmP111	RmG212 with changed wild-type *pstC*	This study
RmP371	RmG490 with changed wild-type *pstC*	This study
RmP379	Rm5000, truncated *pstC1021* allele	This study
RmP385	RCR2011 *phoC*490::ΩSp	This study
RmP388	Rm5000, *phoCDET* deletion	This study
RmP559	RCR2011 *phoB3*::Tn*V*	This study
RmP626	RmP110 *phoB3*::Tn*V*	This study
RmP633	RCR2011 *pit310*::Tn*5 phoC490*::ΩSp	This study
RmP636	RmP110 *pit310*::Tn*5 phoC490*::ΩSp	This study
Escherichia coli strains		
MT616	MT607 (pRK600), mobilizer	24
MT620	MT607 Rif-20	24
DH5α	ϕ80d*lacZ*Δ*M15*	GIBCO BRL
BL21 (DE3)/pLysS	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)/pLysS (Cm^r)	Novagen
Phage		
ϕM12	*S. meliloti* transducing phage	25
Plasmids		
pAB2001	pUC6S with *lacZ-aacC1* cassette, Amp^r	9
pK18/19GIImob	Suicide vector, *gusA*, Km-Nm^r, *mob*	34
pJB3J1	R68.45 derivative, Tc^r Km^s	Lab stock
pPH1JI	IncP broad-host-range plasmid, Gm^r Sp^r Cm^r	Lab stock
pHP45Ω	pBR322 Ap^r containing Sm^r Sp^r Ω cassette	52
pUC119	pUC18 derivative, clone vector	Lab stock
pJQ200-SK	*sacB* suicide vector	54
pTH659	pK18GIImob with 2.7-kb HindIII/EcoRI fragment of *pstAB*	This study
pTH663	pK18GIImob, 2.7-kb *pstAB, pstB*::*lacZ-aacC1* (SmaI)	This study
pTH664	pK18GIImob, 2.7-kb *pstAB, pstB*::*lacZ-aacC1* (SmaI)	This study
pTH665	pK18GIImob, 2.7-kb *pstAB, pstB*::ΩSp (SmaI)	This study
pTH691	pUC119, 7.5-kb HindIII fragment of R-prime plasmid	This study
pTH825	680-bp PhoB coding sequence in pET21a (NdeI-HindIII)	This study
pTH1002	pTH1582, XhoI-EcoRI of *phoR-pstS* intergenic region	This study
pTH1457	C-terminal 312 bp of PhoB in pET21a (NdeI-HindIII)	This study
pTH1582	pJP2(51) with replaced *gusA* from pFUS1	77
pTH1734	*phoC*::*gusA* fusion in pTH1582	This study
pTH1735	*orfA-pit*::*gusA* fusion in pTH1582	This study
pTH1736	*pstS*::*gusA* fusion in pTH1582	This study
pTH1892	*pstS* promoter and coding sequence in pGEM-T (Promega)	This study
pTH1906	pJQ200-SK, 1.4-kb PstI-EcoRI fragment of *pstC1021* allele	This study
pTH1907	pTH1906, *pstC1021* changed into wild-type *pstC*	This study

[a] APase$^+$, alkaline phosphatase positive; APase$^-$, alkaline phosphatase negative.

Phosphate transport assays. The P_i uptake assay was performed essentially as described previously (69). *S. meliloti* strains were grown in LBmc overnight to OD_{600} of around 1.0, washed three times in P_i-free MOPS-buffered medium, and subcultured 1:50 into MOPS minimal medium with or without phosphate. Cells were grown for 10 to 12 h, harvested, washed once in P_i-free MOPS medium, and resuspended in MOPS medium to an OD_{600} of around 1.0 for strains with the *pstC* wild-type allele and to OD_{600} of around 10.0 for strains carrying the *pstC1021* allele. Thirty microliters of cells was added to 450 μl of P_i-free MOPS medium and equilibrated for 5 min at 30°C in a water bath. Twenty-microliter portions of various concentrations of ^{33}P-labeled K_2HPO_4 ([^{33}P]orthophosphoric acid prepared with a specific activity of 135 Ci/mol; NEN Research Products) was added to initiate P_i transport. Aliquots (100 μl) were removed from the assay mixture at different times, placed on nitrocellulose filters (pore size, 0.45 mm; HAWP 025 00; Millipore, Bedford, Mass.) that had been presoaked in 1 M

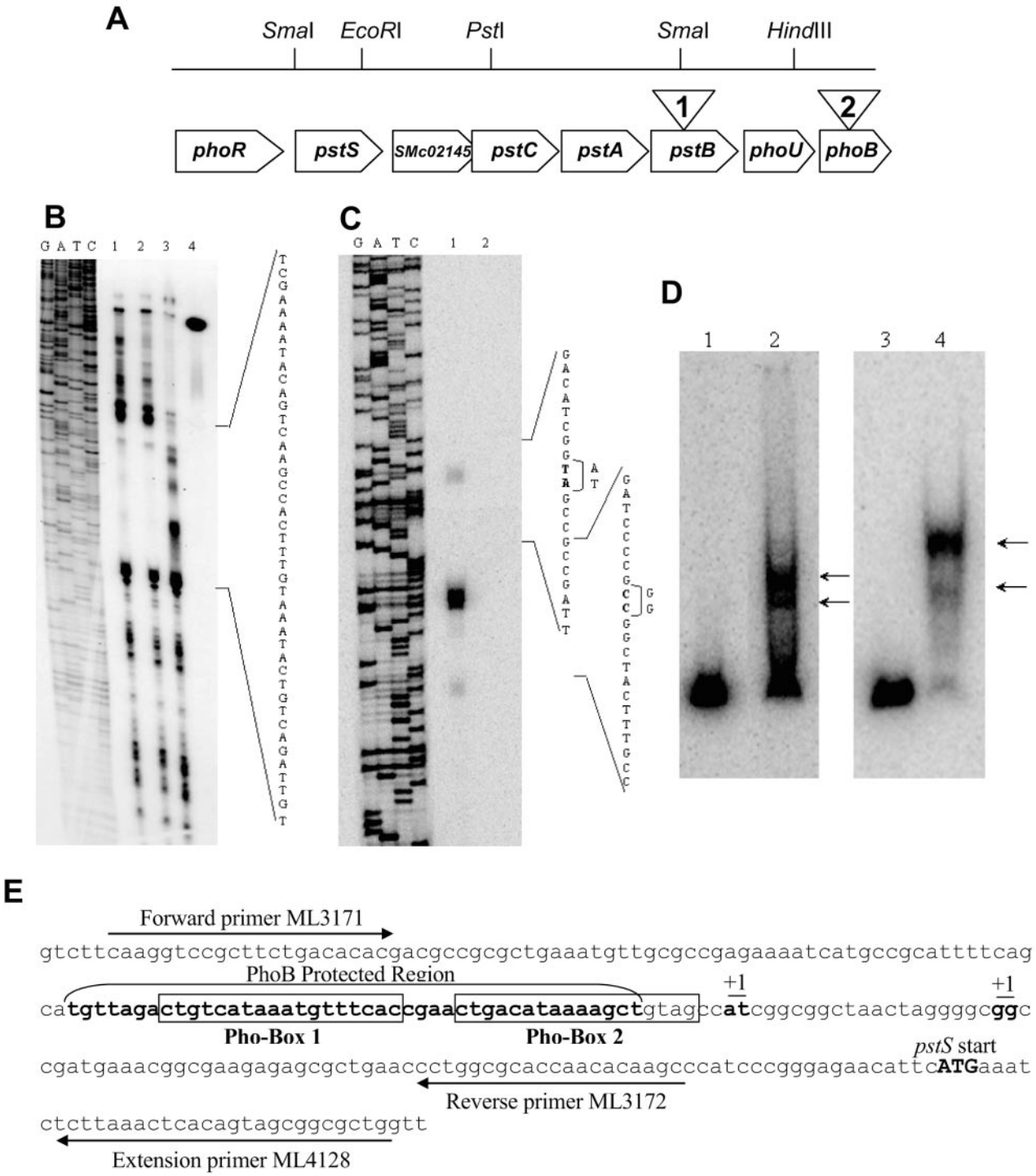

FIG. 1. (A) Schematic diagram of the gene cluster that is between nucleotide positions 565,044 and 573,164 on the *S. meliloti* 1021 chromosome. The SMc02146 gene is referred to as *pstS*. Triangle 1 indicates the position of the *lazZ-aaC1* or ΩSp cassettes in the *pstB* fusion and insertion mutant strains RmK385, RmK386, RmK390, and RmK426, whereas triangle 2 indicates the location of the *phoB*::Tn*V* insertion in the RmK426 strain (see Materials and Methods). (B) DNase I footprint of the *pstS* promoter. Lane 1, PhoB protein incubated with 191-bp PCR product of ^{32}P-labeled forward primer and unlabeled reverse primer and digested with DNase I; lane 2, PhoB^{DBD} protein incubated with 191-bp PCR (primers ML3171 and ML3172) product and digested with DNase I; lane 3, PCR product digested with DNase I; lane 4, undigested PCR product. The DNA sequence ladder was generated by using forward primer ML3171 and the pTH1892 plasmid as the template. (C) Determination of transcriptional start sites of the *pstS* promoter in *S. meliloti* strain Rm1021 through extension of ^{32}P-labeled ML4128 primer. Lane 1, RNA obtained from Rm1021 cells grown in LB medium; lane 2, RNA obtained from RmH615 (Rm1021 *phoB*) cells grown in LB medium. (D) Electrophoretic mobility shift assays of 191-bp PCR product (primers ML3171 and ML3172) containing *pstS* promoter region. Lane 1, 191-bp ^{32}P-end-labeled PCR; lane 2, labeled probe incubated with 100 ng full-length PhoB protein; lane 3, labeled probe; lane 4, labeled probe incubated with 50 ng PhoB^{DBD}. (E) Sequence of *pstS* promoter region, showing the locations of Pho boxes, the DNase I protected region, transcription initiation sites, and the primers used.

K_2HPO_4/KH_2PO_4 (pH 7.0), and immediately washed with P_i- and carbon-free MOPS medium. The filters were dried, placed in scintillation liquid (BCS; Amersham, Little Chalfont, England), and counted. For competition experiments, cells were equilibrated for 4 min at 30°C before 4 μM or 40 μM inhibitor was added (1 min before [^{33}P]orthophosphate was added at a final concentration of 1 μM). All the transport assays were performed in triplicate, and the values reported below are the means of three assays.

Isolation of total RNA and primer extension analysis. Total RNA was prepared as described by MacLellan et al. (39). Briefly, overnight cultures of *S. meliloti* Rm1021 and RmH615 (*phoB3*::Tn*V*) (5) were used to inoculate 100-ml portions of LBmc. Cultures were grown with shaking at 30°C to OD_{600} of 0.4 to 0.6. Without delay, cultures were supplemented with 0.1 volume of cell stop solution (39) and immediately centrifuged to pellet the cells. The cell pellets were flash frozen in liquid nitrogen and stored at −80°C until use. Thawed pellets from 50 ml of culture were resuspended in 960 μl of RNase-free water and split into two portions (480 μl each). Cells were lysed by addition of an equal volume of hot phenol buffer (39) at room temperature, and each suspension was vortexed vigorously and heated at 95°C for 1 min. The lysed cell suspension was centrifuged for 10 min at high speed to pellet debris, and the aqueous supernatant was subjected to two phenol-chloroform extractions (using a 1:1 ratio of unbuffered phenol and chloroform) and one final chloroform extraction. Nucleic acids were precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of isopropyl alcohol on ice for 30 min. DNA in the sample was removed by digestion with RNase-free DNase I, and RNA was recovered after phenol-chloroform extraction by precipitation as described above.

To identify the transcriptional start site through primer extension, a 27-mer oligonucleotide primer (5′-CCAGCGCCGCTACTGTGAGTTTAAGAG-3′) complementary to nucleotide positions 8 to 34 of the protein coding region was end labeled with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase (New England Biolabs) at 37°C for 45 min, followed by removal of unincorporated label by passage through a QIAGEN oligopurification column. In a typical primer extension reaction, ~40 μg of total *S. meliloti* RNA was supplemented with 2×10^5 cpm of end-labeled primer, 4 μl of 5× reverse transcriptase buffer, 0.8 μl of a deoxynucleoside triphosphate mixture containing all four nucleotides (25 mM each), and enough RNase-free water to bring the volume to 16 μl. The mixture was heated at 65°C in a water bath for 10 min, allowed to cool slowly to room temperature, and then placed on ice. The annealed mixture was supplemented with 2 μl of 100 mM dithiothreitol (DTT) and 1 μl of RNaseOUT (Invitrogen) and was incubated at 50°C for 2 min before addition of 1 μl (200 U) of SuperScript III reverse transcriptase (Invitrogen) and further incubation at 50°C for 50 min. The reaction was stopped with an equal volume of formamide containing 2× loading dye. The primer extension product was loaded onto a 6% acrylamide–7 M urea sequencing gel and run alongside a sequencing ladder generated by using the same primer with plasmid template pTH1892 (1.25-kb PCR product containing the *pstS* coding region and the upstream promoter region in the pGEM-T TA cloning vector from Promega and a Sequenase version 2 DNA sequencing kit [Amersham]).

Overexpression and purification of PhoB and PhoB^{DBD}. The *S. meliloti* PhoB protein coding region and PhoB DNA binding domain (23) and the PhoB^{DBD} (amino acids 126 to 227) coding sequences were PCR amplified using primers ML1636 (forward) (CGAGTTACCATATGTTGCCGAAGATTGCCG) and ML1637 (reverse) (CGTAAGCTTGCTCTCCAGCGAATAGCCC) and primers ML2723 (forward) (GGAATTCCATATGGAGGTTCTGTCGACGCTCCTG) and ML2724 (reverse) (CGTAAGCTTGCTC TCCAGCGAATAGCCC), respectively, and cloned into NdeI and HindIII restriction sites of the pET21a vector (Novagen). The resulting plasmids, pTH825 and pTH1457, were transformed into BL21(DE3)/pLysS to develop the J841 and M341 bacterial strains for PhoB and PhoB^{DBD}, respectively. Cultures were grown in LB medium containing 100 μg/ml of ampicillin and 30 μg/ml of chloramphenicol at 37°C until the OD_{600} was ~0.5 and induced with 0.3 to 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 30°C. Proteins were purified on Ni-nitrilotriacetic acid resin (QIAGEN) used according to manufacturer's instructions. Eluted fractions containing purified proteins were pooled and dialyzed against 50 mM HEPES (pH 8.0)–300 mM NaCl–10% glycerol–1 mM DTT. The purities of the two proteins were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and quantities were measured using the Bio-Rad dye reagent.

DNase I footprinting. DNase I footprinting assays were based on the method described by Schmitz and Galas (60), with modifications. The target DNA fragment from the *pstS* promoter was obtained by PCR amplification with 5′-^{32}P-labeled forward primer ML3171 (CAAGGTCCGCTTCTGACACAC) and unlabeled reverse primer ML3172 (GCTTGTGTTGGTGCGCCAGG), using pTH1892 as the template, which yielded a 191-bp DNA fragment. The labeled PCR product was purified using QIAGEN PCR purification columns to remove unincorporated [γ-^{32}P]ATP. DNase I footprinting was performed by incubating ~50,000 cpm of labeled PCR product with protein binding buffer [20 mM HEPES (pH 8.0), 5 mM magnesium acetate, 80 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM $CaCl_2$, 200 μg/ml bovine serum albumin, 100 ng of poly(dI-dC), and 2% glycerol), 250 ng of full-length PhoB protein or 125 ng of PhoB^{DBD} protein, and 0.25 U of DNase I enzyme (Invitrogen) for 1 min. Reaction products were resolved on an 8% acrylamide–7 M urea sequencing gel alongside a sequencing ladder generated by using the ML3171 primer with plasmid template pTH1892.

Electrophoretic mobility shift assay. The basic gel shift protocol used was based on the methods of Garner and Revzin (28). The DNA probe was derived from a 191-bp PCR product of primers ML3171 and ML3172 (see above). This PCR product was labeled at both ends using [γ-^{32}P]ATP and T4 polynucleotide kinase and was subsequently purified with a QIAGEN PCR purification column to remove unincorporated label. Protein-DNA binding reaction mixtures contained approximately 6,000 cpm DNA probe in 15 μl (final volume) of DNA binding buffer [20 mM HEPES (pH 8.0), 5 mM magnesium acetate, 50 mM KCl, 0.5 mM EDTA, 2 mM dithiothreitol, 200 μg of bovine serum albumin/ml, 100 ng of poly(dI-dC), and 4% glycerol] with or without 50 to 100 ng of purified PhoB or PhoB^{DBD}. The reaction mixtures were incubated on ice for 10 min, and this was followed by 20 min of incubation at 25°C. The reaction mixtures were resolved onto nondenaturing 5 to 6% polyacrylamide gels in 0.5× Tris-borate-EDTA at room temperature. Following electrophoresis, the probes were detected either using a Storm820 phosphorimager (Amersham Pharmacia Inc.) or by radiography.

RESULTS

Expression of *pstSCAB-phoUB* in Rm1021. Strain Rm1021 is a streptomycin-resistant derivative of *S. meliloti* wild-type strain Rm2011 (43). Rm2011 and RCR2011 are alternative laboratory names given to nodule isolate SU47, which was originally isolated in 1939 in New South Wales, Australia (68).

Rm1021 grows well in MOPS-buffered minimal medium containing 2 mM P_i, while an Rm1021 *phoC* mutant and a *phoC pit* double mutant grow very poorly in a medium containing 2 mM P_i (4, 5, 6). While these data suggested that the *phoCDET* and *orfA-pit* genes encode the major P_i transporters in *S. meliloti*, the sequence of the *S. meliloti* chromosome upstream of the *phoUB* genes revealed a gene cluster with similarity to the *pstSCAB* P_i transport genes of *E. coli*, and this prompted us to investigate this region further. This gene cluster lies at nucleotides 565,258 to 573,066 on the *S. meliloti* 1021 chromosome and includes the genes annotated as *phoR*, SMc02146, SMc02145, *pstC*, *pstA*, *phoU*, and *phoB* (Fig. 1A) (14). SMc02146 is similar in terms of sequence and gene location to the previously annotated phosphate-binding periplasmic proteins (PstS) in many organisms, and below we refer to the SMc02146 gene as *pstS*. SMc02145 is annotated to encode a 201-amino-acid signal peptide hypothetical protein, and this open reading frame overlaps the *pstC* gene by 31 nucleotides. PstC and PstA are annotated as phosphate transport permeases, and PstB is the transport ATP binding protein. PhoU is predicted to be involved in regulation, and PhoB is the response regulator believed to be activated by phosphorylation by its cognate sensor protein PhoR (40, 41, 42, 65).

In initial experiments, we constructed a *pstB*::ΩSp^r insertion mutant and found that the mutation abolished alkaline phosphatase activity in cells grown under P_i-limiting conditions. This suggested that *pstB* or a gene(s) downstream of *pstB* (such as *phoB*) was required for P_i-regulated alkaline phosphatase synthesis (Fig. 1A). To address this issue and also to monitor expression of the *S. meliloti pstSCAB* gene cluster, we constructed the nonpolar chromosomal *pstB*::*lacZ-aacCI* gene fu-

sion strain RmK385, in which transcription of genes downstream of *pstB* was driven from the *aacCI* promoter (see Materials and Methods). Cultures of this strain grown in MOPS medium with 2 mM P_i had high β-galactosidase activities (~160 Miller units) and low alkaline phosphatase activities (<5 Miller units), while cultures grown in MOPS medium with no added P_i had both high β-galactosidase activities (~160 Miller units) and high alkaline phosphatase activities (50 Miller units). Moreover, disruption of *phoB* (*phoB3*::Tn*V*) in the *pstB::lacZ-aacCI* strain was found to eliminate β-galactosidase activity (<7 Miller units) and alkaline phosphatase activity (<5 Miller units). These data revealed that expression of alkaline phosphatase in Rm1021 required PhoB and was responsive to the concentration of available P_i and that *pstB* expression required PhoB but paradoxically was unresponsive to P_i in the medium.

Identification of PhoB binding sites in the *pstS* promoter. Since *pstB* transcription appeared to be dependent on PhoB, we searched the *phoR-phoB* DNA region for motifs with sequence similarity to the PhoB binding sites, as described previously for *E. coli* (35). Two such sites were located upstream of the *pstS* gene (Fig. 1E). To investigate the functionality of these sites, we cloned a DNA fragment made up of sequences 250 bp upstream of the *pstS* translation start codon and 30 bp downstream into the reporter plasmid pFUS1 (56). β-Glucuronidase activity from this plasmid was found to be expressed constitutively (400 Miller units) in Rm1021 cells grown in the presence of high and low P_i concentrations, but only background activity (20 Miller units) was observed in the Rm1021 *phoB* mutant strain, RmH615. Thus, *pstS* transcription in Rm1021 appears to be unresponsive to the P_i concentration in the medium, yet *pstS* transcription required the *pho* regulator PhoB.

The nature of the PhoB-like binding sites was examined further in DNA gel shift experiments. For these experiments, we employed a full-length PhoB protein with a C-terminal His tag and a truncated protein consisting of PhoB C-terminal amino acids 125 to 227 followed by six histidine residues (70). In gel shift experiments with a 191-bp DNA fragment from the *pstS* promoter, these proteins produced two distinct products with reduced mobility in polyacrylamide gels (Fig. 1B). To investigate the site(s) to which PhoB bound, DNase I footprinting of the *pstS* promoter region in the presence and absence of PhoB or $PhoB^{DBD}$ was performed (Fig. 1C). Both PhoB and $PhoB^{DBD}$ were found to protect a 50-nucleotide region that included all of predicted PhoB binding site I and all but four nucleotides at PhoB site II (Pho box 1 and 2 sequences in Fig. 1E). The *pstS* promoter was subsequently mapped by primer extension with mRNA from Rm1021 cells (Fig. 1C). A prominent extension product was detected, and analysis of the DNA sequence revealed that this site was 45 nucleotides downstream of the first of the two predicted PhoB binding sites. In summary, data from the analysis of the *pstS-phoB* gene cluster in Rm1021 suggested that this region was transcribed as an operon with a single *pstS* promoter that was PhoB dependent but, unusually, was apparently not responsive to changing P_i concentrations in the medium.

***pit phoC* suppressor mutation restores a full-length reading frame to the *pstC* gene in Rm1021.** Examination of the symbiotic phenotype of the *S. meliloti pit phoC* double mutant (RmG830) revealed that it formed small white nodules that failed to fix nitrogen (Fix^-). This was expected as Rm1021 *phoC* mutants alone are Fix^- (4, 16). Thus, when grown in the absence of fixed nitrogen, alfalfa plants inoculated with RmG830 were stunted and yellow and died about 5 weeks after inoculation. However, among 30 such plants, a single green plant was observed, and the roots of this plant had a single pink nodule. Isolation and characterization of bacteria from this nodule revealed that they contained the antibiotic resistance markers (Nm^r and Sp^r) associated with the *pit310*::Tn*5* and *phoC490*::ΩSp insertion mutations of the RmG830 inoculant strain. Moreover, when alfalfa seedlings were inoculated with the purified nodule isolate (designated RmP101), the resulting plants formed pink nitrogen-fixing root nodules that appeared to be indistinguishable from wild-type Fix^+ nodules. In addition, the nodule isolate RmP101 grew like the wild type in MOPS medium containing 2 mM P_i. These results suggested that this strain contained a suppressor mutation that restored P_I uptake and N_2-fixing ability to the *phoC pit* double mutant. To investigate this further, the SMc02145-*pstC* intergenic region was amplified by PCR from suppressor strain RmP101 and its parent strain RmG830 (see Materials and Methods). The sequences of this region showed that the RmG830 sequence was identical to the annotated Rm1021 GenBank sequence. However, examination of the suppressor mutant sequence showed that it differed from the Rm1021 and RmG830 sequences by a single G insertion between nucleotides 512 and 513 of the annotated 606-bp SMc02145 gene. This single-nucleotide addition to the SMc02145 sequence altered the amino acid reading frame from residues 171 to 201, and the new reading frame was in frame with the annotated *pstC* gene. The resulting new *pstC* gene was 1,485 nucleotides long and encoded a 494-amino-acid PstC protein (Fig. 2).

These data led us to suspect that this locus may contain a mutation, and detailed examination of this region revealed that the SMc02145 gene (nucleotides 567841 to 568443), annotated as a gene encoding a hypothetical signal peptide protein, had a 31-bp overlap with the 5′ end of the *pstC* gene (nucleotides 568413 to 569324). Moreover, searches of the GenBank database with the SMc02145 sequence revealed similarity between it and sequences encoding the N-terminal region of PstC proteins from various bacteria. This suggested that SMc02145 and *pstC* may be a single gene but that a mutation resulted in their annotation as two distinct genes (Fig. 2). Comparison of the sequence of the SMc02145-*pstC* gene region from RCR2011, following its amplification by PCR, revealed that RCR2011 and Rm1021 differed by a single C addition between nucleotides 552 and 553 of the reported Rm1021 SMc02145 sequence (Fig. 2). The C deletion mutation in Rm1021 was also recently reported by Krol and Becker; however, these authors did not establish that the Pho regulon phenotypes examined resulted from the deletion mutation (36). As in the case of suppressor strain RmP101, the reading frame deduced from the RCR2011 sequence, which contained the single C nucleotide insertion, fused the SMc02145 and *pstC* genes to generate a PstC protein containing 494 amino acid residues. The sequences of the predicted RCR2011 and RmP101 PstC proteins differed over a 13-amino-acid region as the G insertion in RmP101 occurred 42 nucleotides upstream of the C deletion mutation (Fig. 2).

Amplification of the SMc02145-*pstC* region from the Lac^- Rm1021 derivative RmG212 revealed that its sequence was the

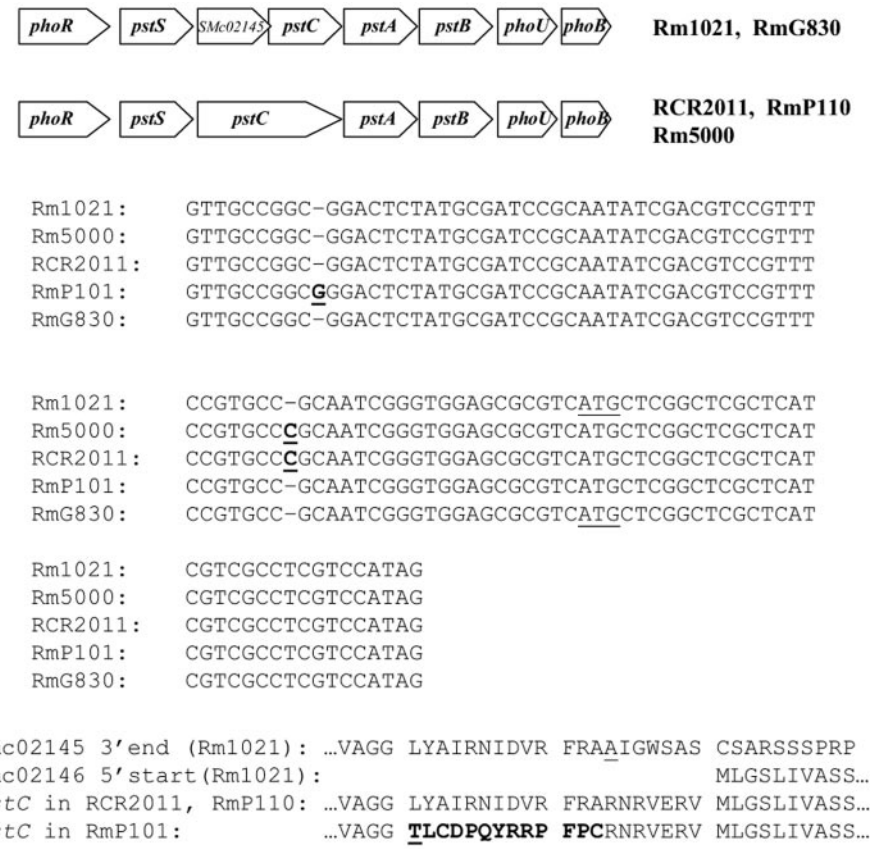

FIG. 2. Nucleotide and amino acid sequences showing the 3′ 105-nucleotide region from SMc02145 and 34 nucleotides from the 5′ region of the *pstC* gene in *S. meliloti* 1021 and its derivative RmG830 (Rm1021 *pit310*::Tn*5* *phoC490*::ΩSp). Schematic diagrams of the *pstSCAB* region with the annotated SMc02145 gene and truncated *pstC* gene in strain Rm1021 and its derivative RmG830 are at the top; also shown is the position of the full-length *pstC* gene in wild-type strain RCR2011, Rm5000, and RmP101 (Fix^+ derivative of RmG830). The aligned nucleotide sequences with the C nucleotide (boldface type and underlined) present in RCR2011 and Rm5000 but absent in Rm1021 and its derivatives are shown. The single G insertion in the RmP101 sequence is also indicated by boldface type and underlining. In the translated sequences for the Rm1021 SMc02145 and SMc02146 proteins (PstC′) and PstC from RCR2011 (similar to Rm5000) and the sequence resulting from the G frameshift mutation in RmP101, the amino acids that differ from those in RCR2011 are indicated by boldface type. The underlined ATG indicates the translation start site for the truncated *pstC* gene in Rm1021 and RmG830.

same as that of Rm1021. On the other hand, the sequence of this region obtained from the rifampin-resistant SU47 derivative Rm5000 was the same as the sequence obtained from RCR2011; i.e., it contained an additional C compared with the Rm1021 sequence. Thus, Rm1021 and its derivatives carry a C deletion mutation that generates a truncated PstC protein and the SMC02145 protein. Below we refer to the *pstC* allele in Rm1021 as *pstC1021*.

Confirmation and extent of the Pho phenotype resulting from the *pstC1021* deletion mutation. To confirm that the C deletion mutation in Rm1021 alone was responsible for the Pho phenotype of this strain, we tried to correct the mutation in Rm1021 by site-directed mutagenesis. Accordingly, a PstI-EcoRI fragment carrying the Rm1021 *pstS-C* region was cloned into the gentamicin-resistant *sacB* suicide plasmid pJQ200-SK (54) to obtain pTH1906. Oligonucleotides ML3567 and ML3568 were then employed to convert the *pstC1021* mutation to the wild type, which yielded plasmid pTH1907. Single-crossover Sm^r Gm^r recombinants were selected following transfer of pTH1907 to Rm1021, RmG212 (Rm1021 Lac^-), and RmG490 (Rm1021 *phoC490*::ΩSp). Resolution of the cointegrate by recombination resulted in loss of the plasmid, and a proportion of the Gm^s recombinants carried the additional C residue of the wild-type *pstC* allele, as identified by PCR amplification and sequencing of this region from Gm^s recombinants. In a similar manner, plasmid pTH1906 was used to introduce the *pstC1021* allele into Rm5000. Following purification of Rif^r Gm^r transconjugant cointegrates, Gm^s recombinants that carried the *pstC1021* allele, such as RmP379, were identified following PCR amplification and sequencing. The Pho phenotype of the resulting strains was assessed by measuring *pstS* promoter activity (see below) and by measuring the alkaline phosphatase activity in cultures grown for 12 h in LBmc. Strains carrying the *pstC1021* allele had high alkaline phosphatase activities, whereas very low activities were observed in strains carrying the "wild-type" *pstC* locus. Thus, Rm1021 and the *pstC1021* derivative of Rm5000 (RmP379) had 29.6 ± 2.1 and 27.5 ± 2.6 U of alkaline phosphatase activity, respectively, whereas the $pstC^+$ derivative of Rm1021, RmP110, and Rm5000 had 4.9 ± 1.0 and 2.6 ± 0.4 U of activity, respectively. The alkaline phosphatase activity of RmG490 (Rm1021 *phoC490*::ΩSp) was very high (48.4 ± 3.0 U); however, the alkaline phosphatase

TABLE 2. Expression of a plasmid-borne *pstS*::*gusA* gene fusion in *S. meliloti* strains carrying *pstC1021* or *pstC* wild-type alleles following growth in minimal medium with limiting and excess P_i

Strain (properties)	β-Glucuronidase activity (Miller units)[a]	
	P0 medium	P2 medium
RCR2011 (empty vector)	40 ± 16	41 ± 22
RCR2011 (*pstC*$^+$, pTH1736)	1,286 ± 59	69 ± 21
Rm1021 (*pstC1021*, pTH1736)	1,000 ± 64	1,076 ± 83
RmP110 (Rm1021, *pstC*$^+$, pTH1736)	1,208 ± 70	73 ± 28
RmH615 (Rm1021, *phoB*, pTH1736)	53 ± 30	118 ± 16

[a] The *pstS*::*gusA* fusion plasmid was pTH1736. The β-glucuronidase activity of *S. meliloti* cells grown in phosphate-free MOPS minimal medium (P0 medium) or MOPS minimal medium containing 2 mM phosphate (P2 medium) was measured as described in Materials and Methods. RCR2011 carrying the empty reporter plasmid pTH1582 was used as a background control. The data are averages ± standard errors for triplicate determinations.

activity was dramatically lower in the *phoC pstC*$^+$ derivative RmP371 (2.3 ± 0.3 U).

To investigate the influence of the chromosomal *pstC* allele on transcription from the *pstS* promoter, plasmid pTH1002 carrying a *pstS-gusA* fusion was introduced into RCR2011, RmP110, and Rm1021, and β-glucoronidase activity was measured in cells cultured in MOPS media with 2 mM P_i and no added P_i (Table 2). As indicated above, in the Rm1021 (*pstC1021*) background, *pstS-gusA* was transcribed at a high level in cells grown with excess or limiting P_i, and this transcription was PhoB dependent as only background activity was detected in the *phoB* mutant RmH615. However, in RCR2011 and RmP110, which carried the wild-type *pstC* allele, *pstS* transcription was highly induced in P_i-starved cultures, whereas very low β-glucoronidase activity and hence little *pstS* transcription were detected in cultures grown with excess P_i (Table 2). Thus, the presence of the *pstC1021* allele appeared to override the dependence of *pstS* transcription on the availability of P_i in the medium.

Fix$^-$ phenotype of *phoC* mutations is dependent on the *pstC1021* allele. The effects of the *pstC1021* and *pstC*$^+$ alleles on the alkaline phosphatase activity of *phoC* mutant cells prompted us to investigate the effects of *pstC* alleles on the symbiotic Fix$^-$ phenotype of *phoC* mutant cells. Thus, *phoC* mutant strains and derivatives that carried the wild-type *pstC*$^+$ and *pstC1021* alleles were inoculated onto alfalfa seedlings. Their symbiotic N_2 fixation phenotypes were measured by determining the plant dry weight following growth for 28 days in Leonard jars under nitrogen-deficient conditions (Table 3). The dry weights of plants inoculated with *pstC*$^+$ *phoC* strains were the same as the dry weights of plants inoculated with Fix$^+$ parent strains RCR2011, Rm1021, and Rm5000. This contrasts with the Fix$^-$ phenotype of plants inoculated with the Rm1021 (*pstC1021*) *phoC* mutant strains RmG490 and RmG830, whose dry weights were similar to those of the uninoculated control plants which failed to fix nitrogen (Table 3). We concluded that the Fix$^-$ phenotype resulting from *phoC* mutations depended on the presence of the *pstC1021* mutation. Moreover, the slow growth of RmG490 (Rm1021 *phoC490*::ΩSp) in MOPS-buffered minimal medium containing 2 mM P_i was also dependent on the *pstC1021* allele since RmP636 and RmP371 grew like the wild type in medium containing 2 mM P_i.

TABLE 3. Symbiotic phenotypes of *S. meliloti* strains[a]

Strain	Relevent genotype	Dry wt (mg/plant)	%
RmG490	Rm1021 *phoC490*::ΩSp *pstC1021*	7 ± 3	15
RmP371	Rm1021 *phoC490*::ΩSp, *pstC* corrected	43 ± 3	92
Rm1021	Wild type, *pstC1021*	47 ± 5	100
RmP110	Rm1021, *pstC* wild-type allele (corrected)	44 ± 3	92
Control	No inoculation	3 ± 1	7
RmG830	Rm1021 *phoC490*::ΩSp *pit310*::Tn*5* *pstC1021*	3 ± 1	6
RmP101	Fix$^+$ suppressor mutant of RmG830	48 ± 4	101
RmP636	Rm1021 *phoC490*::ΩSp *pit310*::Tn*5*, *pstC* wild type	49 ± 5	103
RmP385	RCR2011 *phoC490*::ΩSp	45 ± 1	96
RCR2011	Wild type	50 ± 4	106
RmP633	RCR2011 *phoC490*::ΩSp *pit310*::Tn*5*	52 ± 3	111
RmP379	Rm5000, *pstC1021* allele	52 ± 2	110
RmP388	Rm5000, *phoCDET* deletion	45 ± 2	95
Rm5000	Derivative of wild-type strain SU47, Rif resistance	46 ± 5	99

[a] Plants were harvested 28 days after inoculation. The dry weights are shoot dry weights (averages ± standard errors for three determinations with 10 plants each). The percentages were determined by comparison with the value for the wild-type strain (Rm1021).

What is the biochemical role of *pstSCAB*? The effects of *pstC1021* and the wild-type *pstC* allele on the growth phenotype of *phoC* mutant strains suggested that the wild-type *pstSCAB* locus encodes a P_i transport system. We assumed that the partial function of this system allowed sufficient P_i uptake to enable *phoC pit* double mutants to grow, albeit slowly, in MOPS-buffered minimal medium containing 2 mM P_i (6). To investigate this further, we constructed a *phoC pit* double mutant carrying the wild-type *pstSCAB* genes. The resulting strain, RmP636, grew like parent strains RmP110 and Rm1021 in MOPS medium containing 2 mM P_i. In uptake assays with P_i-starved cells and a substrate concentration of 4 μM P_i, $^{33}P_i$ was rapidly transported into the RmP110 *phoC pit* double mutant, RmP636, and into RmP110 (Fig. 3). In contrast to RmP636 and RmP110, which had the wild-type *pstSCAB* locus, the rate of P_i transport into Rm1021 was low. This is consistent with the modest V_{max} (6.8 and 1 to 2 nmol/min/mg protein for P_i transport via the PhoCDET and OrfA-Pit systems, respectively) (6, 69). A time course for P_i uptake into RmG830 (Rm1021 *phoC pit*) and RmP101 revealed that the *pstC1021* frameshift suppressor mutation resulted in substantial P_i transport via the modified PstSCAB transport system (Fig. 3). As might be expected from the 13-amino-acid difference between the PstC sequences in RmP101 and RmP110, the rate of uptake in RmP101 was not restored to wild-type RmP110 levels.

Examination of the kinetics of P_i transport into RmP636 revealed Michaelis-Menton-type kinetics with a K_m for PstSCAB-mediated P_i uptake of 0.2 μM and a V_{max} of 70 nmol/min/mg protein (Fig. 4). The specificity of PstSCAB transport was determined by addition of potential competitors at 4 and 40 times the concentration of labeled P_i. The addition of phosphonates had minimal effects on the rate of P_i transport, whereas the P_i analogue arsenate severely reduced P_i uptake into the RmP636 cells (Table 4). From these data we concluded that PstSCAB encodes a high-affinity, high-velocity P_i transport system and that in contrast to P_i uptake via the

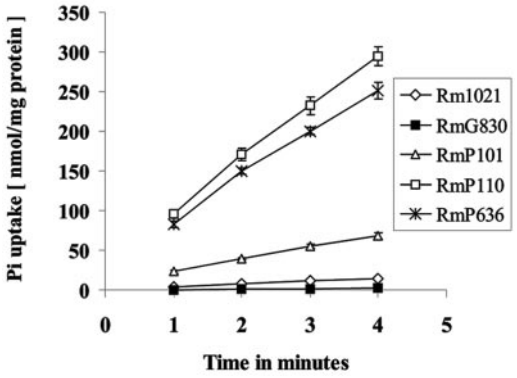

FIG. 3. Time course of $^{33}P_i$ uptake for various *S. meliloti* strains. *S. meliloti* cells were grown as described in Materials and Methods and were subcultured in MOPS medium without added P_i for 10 h prior to the assay. The concentration of P_i in the transport assay buffer was 4 μM. The strains used were Rm1021 (carrying the *pstC1021* allele), RmG830 (Rm1021 *phoC490*::ΩSp *pit310*::Tn*5*), RmP101 (suppressor mutant of RmG830), RmP110 (same as Rm1021 but with the corrected *pstC* allele), RmP636 (RmP110 *phoC490*::ΩSp *pit310*::Tn*5*), and RmP626 (RmP110 *phoB3*::Tn*V*). The symbols indicate the means of triplicate assays. The P_i uptake line for RmP626 (RmP110 *phoB3*::Tn*V*) was similar to the line for RmG830 (the line located on the bottom of the graph), and for clarity the line for RmP626 is not shown.

PhoCDET system (69), phosphonates do not compete for P_i uptake via the PstSCAB system.

Expression of *pit*, *phoC*, and *pstS* in nodules. To monitor expression of the three *S. meliloti* P_i transporters in symbiotic conditions, the promoter regions of *phoC*, *orfA-pit*, and *pstS* were cloned into the modified *parAB*-stabilized pJP2 vector to produce plasmids pTH1734, pTH1735, and pTH1736, respectively. The resulting promoter-*gusA* fusion plasmids were introduced into wild-type strain RCR2011 and its derivative RCR2011 *phoB3*::Tn*V* (RmP559). Expression in the *phoB* mutant was examined since *orfA-pit* expression is negatively regulated by PhoB, while expression of the *pstSCAB* and

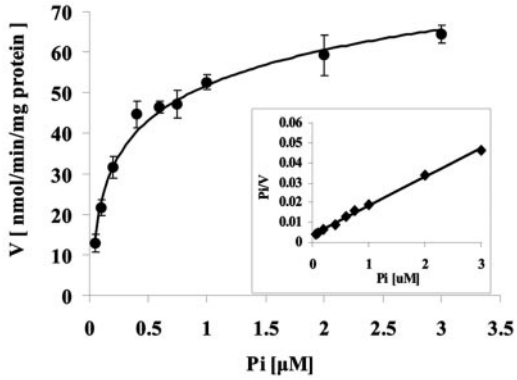

FIG. 4. Kinetics of PstSCAB-mediated $^{33}P_i$ uptake by *S. meliloti*. Strain RmP636 (RmP110 *phoC490*::ΩSp *pit310*::Tn*5*) was subcultured in phosphate-free MOPS minimal medium for 10 h prior to the assay. A Michaelis-Menten plot and a Hanes-Woolf plot for the same data (inset) are shown. The K_m was calculated to be 0.2 μM, and the V_{max} was 70 nmol/min/mg protein. The assays were performed as described in Materials and Methods, and each symbol indicates the mean of triplicate values; the error bars indicate standard errors. The alkaline phosphatase activity for the cells used in this experiment was 60 U.

TABLE 4. Specificity of PstSCAB-directed $^{33}P_i$ uptake

Inhibitor type	Relative phosphate uptake (% activity)[a]	
	4 μM inhibitor	40 μM inhibitor
Control (no inhibitor)	100 ± 6	100.00 ± 3
Ethyl phosphonate	97 ± 3	93 ± 2
Methyl phosphonate	91 ± 1	92 ± 3
Aminoethyl phosphonate	91 ± 1	91 ± 6
KH_2PO_4 (P_i)	11 ± 1	4 ± 2
Arsenate	12 ± 1	4 ± 3

[a] The values are the percentages of phosphate uptake relative to the uptake by the uninhibited control (57 nmol/min/mg protein). All assays were performed with RmP636 (RmP110 *phoC490*::ΩSp *pit310*::Tn*5*) cells cultured in P_i-free MOPS minimal medium for 10 h prior to the assay. Assays were carried out with a P_i concentration of 1 μM, and inhibitors were added to final concentrations of 4 μM and 40 μM. The data are means ± standard errors for triplicate assays. The alkaline phosphatase activity for cells used in this experiment was 70 U.

phoCDET genes requires PhoB. Alfalfa seedlings inoculated with these strains were grown for 4 weeks, and the β-glucuronidase activities in the crude nodule extracts were determined (Fig. 5). In addition, the expression patterns of the three P_i transporters were examined by using histochemical staining for β-glucuronidase in root nodules (Fig. 6). Both assays revealed that the *orfA-pit* system was highly expressed in nodules, while very little *phoCDET* or *pstSCAB* expression was detected, irrespective of whether the nodules were induced by wild-type strain RCR2011 or the RCR2011 *phoB* mutant. The even distribution of the stain in nodules carrying the *orfA-pit*::*gusA* fusion plasmid revealed no zone-specific expression, and neither *phoC* nor *pstS* expression was detected in any nodule zone (Fig. 6). These data strongly suggest that bacteroid metabolism within alfalfa nodules is not P_i limited.

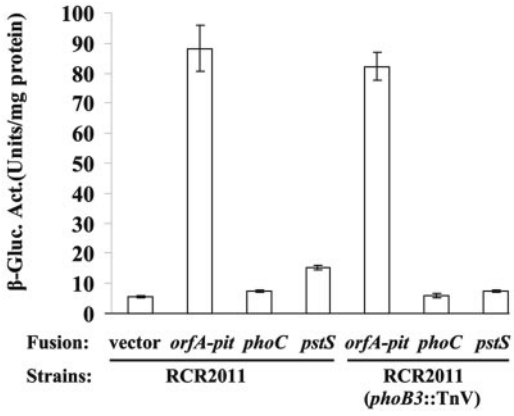

FIG. 5. Expression of *S. meliloti* P_i transporter genes in alfalfa root nodules. The *phoC*, *orfA-pit*, and *pstS* promoter regions were fused to the *gusA* reporter gene in pTH1582 to obtain plasmids pTH1734, pTH1735, and pTH1736, respectively. The plasmid-borne fusions were introduced into wild-type strain RCR2011 and RCR2011 *phoB3*::Tn*V*. The empty vector introduced into RCR2011 was used as a negative control. Nodules were harvested 28 days after inoculation, and β-glucuronidase activities (β-Gluc. Act.) were determined as described in Materials and Methods. Each bar indicates the mean of an assay performed in triplicate, and the error bars indicate standard errors.

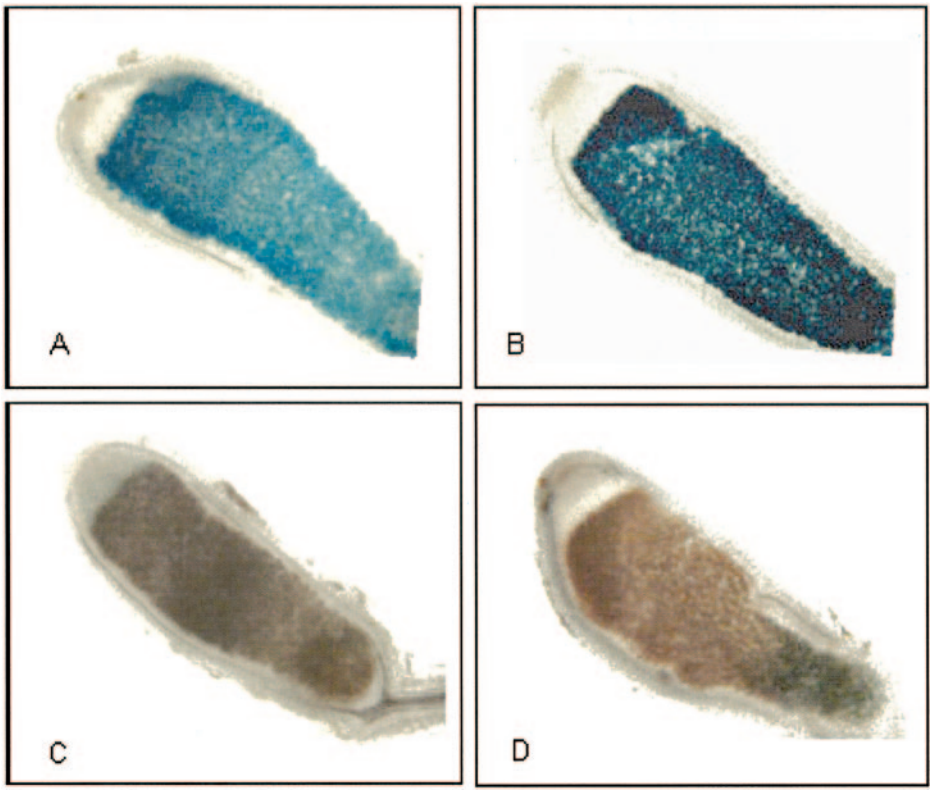

FIG. 6. Photographs of longitudinal sections of 4-week-old alfalfa nodules stained for β-glucuronidase activity. Nodules were obtained from plants inoculated with wild-type strain RCR2011 and its *phoB3*::Tn*V* derivatives containing promoter *gusA* fusions, including RCR2011 (*orfA-pit*::*gusA*) (A), RCR2011 (*phoB3*::Tn*V orfA-pit*::*gusA*) (B), RCR2011 (*pstS*::*gusA*), and RCR2011 (*phoC*::*gusA*) (D). The photographs were taken with a Leitz Laborlux-12 microscope (magnification, 4 × 10) and a Nikon Dxm1200F digital camera system.

DISCUSSION

Our characterization of the *pstSCAB* gene cluster in *S. meliloti* revealed that these genes encode a high-affinity P_i-specific transport system. We examined the kinetics and specificity of PstSCAB-mediated P_i uptake by utilizing a *phoC pit* double mutant in which the wild-type (corrected *pstC* gene) *pstSCAB* genes are expressed. In this strain P_i uptake exhibited Michaelis-Menton kinetics with a V_{max} of 70 nmol/min/mg protein and a K_m of 0.2 μM. The K_m value is similar to that reported for P_i uptake via the *S. meliloti* PhoCDET system and is 10-fold higher than the K_m value of the low-velocity OrfA-Pit system (69). The rate of P_i uptake via PstSCAB was much higher than the rate of P_i uptake via the PhoCDET system, and P_i uptake via the PstSCAB system was not inhibited by methyl or ethyl phosphonates, which is consistent with the *pst* gene designation (*p*hosphate-*s*pecific *t*ransport) (Table 4). To summarize the data in obtained in this and previous studies, *S. meliloti* RCR2011 cells grown in media with excess P_i transport P_i via the low-affinity, low-velocity OrfA-Pit transporter. Upon transfer to P_i-limited media, the *orfA-pit* genes are repressed and the *pstSCAB* and *phoCDET* gene are expressed. P_i transport then occurs via the high-affinity, high-velocity PstSCAB system (Fig. 4).

The identification of the single C deletion mutation in the *pstC* gene of Rm1021 agrees with the recent report of Krol and Becker (36), in which this sequence difference between Rm1021 and Rm2011 (a derivative of RCR2011) was also described. Moreover, employing site-directed mutagenesis, we demonstrated that this specific mutation is responsible for several Pho-related phenotypes observed in the Rm1021 background but not in the RCR2011 background. These phenotypes include the high alkaline phosphatase activity detected when Rm1021 is cultured in LB medium, constitutive *pstS* expression, and the inability of *phoCDET* mutants of Rm1021 to form N_2-fixing root nodules on alfalfa. The Pho phenotype resulting from the *pstC1021* mutation is similar to the Pho regulon constitutive phenotype that results from *pst* mutations in *E. coli* (20, 71, 73) and from mutations in the PHO84 high-affinity P_i transport system in *Saccharomyces cerevisiae* (76). While these data suggest that the transport systems play a role in sensing the P_i concentration and the signal transduction pathway, more recent evidence suggests that it is the intracellular concentration of P_i that is the major signal for regulation of the Pho pathway (3, 32, 76). We note that the *pstC1021* mutation of *S. meliloti* 1021 results in a partial Pho constitutive phenotype in which PhoB transcription of genes such as *pstS* is constitutive (Table 3), whereas transcription of the *phoA* gene encoding alkaline phosphatase and the *phoC* genes is repressed upon addition of 2 mM P_i (5). Our data and conclusions regarding the partial function of the *pstC1021* allele concur with those of Krol and Becker (36). Moreover, our data suggest that members of the Pho regulon show a hierar-

chical regulation that is presumably influenced by the level of activated PhoB protein in the cell. We infer that since the *pstC1021* mutation allows a low level of P_i transport, the resulting Pho phenotype is partial.

We failed to detect P_i uptake via the PstSCAB system in previous studies as the Rm1021 background strain employed in those studies carried the C deletion mutation in *pstC*. While this frameshift mutation at nucleotide position 553 of the 1,485-nucleotide *pstC* gene would be expected to eliminate PstSCAB-mediated P_i uptake, below we discuss several lines of evidence which suggest that a very low level of P_i uptake still occurs via this mutant PstSCAB system. The *pstC1021* mutation is not polar on transcription of the downstream *pstAB-phoUB* genes since *pstB*::*lacZ* gene fusions are expressed in Rm1021 and also PhoB-dependent alkaline phosphatase activity occurs in Rm1021 cells. It is therefore very likely that in Rm1021, the 5′ region of the *pstC* mRNA is translated to give a 201-amino-acid C-terminal truncated protein and that additional translation of the *pstC* mRNA from an internal ATG generates an N-terminal truncated 303-amino-acid PstC protein, as suggested from the genome annotation (26). The lengths of PstC proteins from various organisms, including *E. coli*, *Haemophilus influenzae*, *Bacillus subtilis*, and *Mesorhizobium loti* (294 to 327 amino acids), are similar to the length of the predicted Rm1021 mutant PstC protein, and this protein has six predicted membrane-spanning domains frequently present in the ABC permease proteins (1, 13, 31, 45, 46, 53). Thus, while the size of the predicted wild-type PstC protein (494 amino acids) is similar to the sizes of the annotated PstC proteins from *Agrobacterium tumefaciens* (504 amino acids) and *Brucella suis* 1330 (496 amino acids) (50, 75), it is possible that the N-terminal 150-amino-acid region from PstC is not absolutely required for full PstC function.

We attribute the ability of Rm1021 *pit phoC* mutants to grow, albeit slowly, in media containing 2 mM P_i to be due to residual P_i uptake via the defective *pstSCAB* system. We previously showed that the *S. meliloti phoC pit* double mutant RmG830 could transport $^{33}P_i$ (0.25 nmol/min/mg protein) but that the level of transport was insufficient to allow growth at a wild-type rate in media with 2 mM P_i (16).

Perhaps the most definitive evidence showing that the Rm1021 mutant PstSCAB system has residual P_i uptake activity was obtained from experiments in which we transduced polar and nonpolar *pstB*::*lacZ-aacC1* alleles into Rm1021 *pit*, Rm1021 *phoC*, and Rm1021 *pit phoC* double-mutant backgrounds (Table 5). No recombinants were recovered when either of the *pstB* alleles was transferred into the *pit phoC* double mutant RmG830. However, *pit pstB* double-mutant recombinants were readily constructed with the nonpolar *pstB*::*lacZ-aacC1* allele (RmR385 donor) that expressed *phoB*, while *pit pstB* recombinants were not recovered with the polar *pstB* allele (RmK386 donor) (Table 5). We suggest that the failure to recover *pit pstB* (polar) double mutants is due to the requirement of PhoB for transcription of both the *pstSCAB* and *phoCDET* genes; hence, a *pit pstB* (polar) double mutant is genotypically *pit* and *pstB* and phenotypically PhoCDET$^-$. The data in Table 5 also revealed that we failed to recover *phoC pstB* recombinants with the nonpolar *pstB*::*lacZ-aacC1* allele, while recombinants were readily recovered upon transduction of the nonpolar *pstB* allele into the *phoC* mutant strain (Table 5). This was expected since in a *phoC pstB* (nonpolar) recombinant strain, *phoB* is expressed and the resulting activated PhoB protein represses *orfA-pit* transcription (6). In the *phoC pstB* (polar) recombinants, *phoB* is not transcribed and there is no PhoB protein to repress *orfA-pit* expression; hence, P_i transport occurs via Pit.

TABLE 5. Transduction of polar and nonpolar *pstB* insertion mutations into RmG804, RmG212, RmG490, and RmG830

Recipient	Transduction with donor phage[a]	
	ϕRmK385 (nonpolar *pstB* mutation, *phoB*$^+$)	ϕRmK386 (polar *pstB* mutation, *phoB*)
RmG212 (Rm1021 *lac*)	+	+
RmG804 (Rm1021 *pit310*::Tn*5*)	+	−
RmG490 (Rm1021 *phoC490*::ΩSp)	−	+
RmG830 (Rm1021 *phoC490*::ΩSp *pit310*::Tn*5*)	−	−

[a] +, transductants obtained (frequency, $>1 \times 10^{-6}$/donor); −, no transductants obtained (frequency, $<1 \times 10^{-8}$/recipient). Transductants were selected on LB medium containing gentamicin. RmK385 and RmK386 carry *lacZ-aacC1* cassette insertions in *pstB* (Fig. 1A). In RmK385, transcription of *phoB* is driven from the *aacC1* promoter.

We noted that in the related organism *A. tumefaciens*, disruption of the *phoB* gene appears to be lethal (21). Examination of the *orfA-pit* region in *A. tumefaciens* C58 (*atu4633-4634*) revealed that the Pit-like protein (Atu4633) has a 138-amino-acid C-terminal deletion compared to the 334-amino-acid Pit protein of *S. meliloti* (5, 75). We suggest that in *A. tumefaciens*, the OrfA-Pit transport system is nonfunctional and hence, as in a Pit$^-$ *S. meliloti* strain, disruption of the *phoB* gene would be lethal.

Our finding that the *pstSCAB* system of Rm1021 is partially active for P_i uptake and the transcriptional requirement of this system for *phoB* activation, together with the transduction results shown in Table 5, led us to conclude that the OrfA-Pit, PstSCAB, and PhoCDET systems are the major transport systems that can transport P_i in *S. meliloti*. This is reminiscent of the transport activities reported for *E. coli*, although in *E. coli* the *pit* systems do not appear to be negatively regulated by *phoB* (32, 55, 74). In view of the specificity and activity of the *pstSCAB*-encoded transport system, it is worth reconsidering the annotation of the *phoCDET* genes. These genes were annotated as Pho genes because they are regulated by PhoB and the PhoCDET transport system was demonstrated to transport P_i at a high rate (4, 69). Phylogenetically, the Pho genes cluster with the phosphonate uptake (*phn*) genes (33, 44), and while we demonstrated that there is P_i uptake via the PhoCDET system, the uptake was inhibited by stoichiometric concentrations of alkyl phosphonates, such as methyl or ethyl phosphonates. The *S. meliloti* genome sequence revealed that genes whose sequences are similar to the sequences of the *phnMN* genes from *E. coli* are 1 kb downstream of the *phoT* gene. For these reasons we suggest that the *phoCDET* genes should be reannotated as the *phnCDET* genes.

We previously reported that *phoCDET* mutations eliminate N_2 fixation; however, as described here, it is now clear this Fix$^-$ symbiotic phenotype is dependent on the *pstC1021* mutation, which severely reduces PstSCAB-mediated P_i uptake in *S. meliloti* Rm1021. This finding is consistent with our previous reports in which the symbiotic Fix$^-$ phenotype of Rm1021 *phoC-*

DET mutants was shown to be suppressed to Fix^+ by mutations that allow expression and P_i transport via the OrfA-Pit transport system (5, 48, 69). We have also observed that a *pstB*::ΩSp mutation suppressed the symbiotic Fix^- phenotype of Rm1021 *phoCDET* mutants and the growth deficiency phenotype of the Rm1021 *phoCDET* mutant in the presence of 2 mM P_i (data not shown). This was expected because the *pstB*::ΩSp mutation was polar on the downstream genes, including *phoB*, and *phoB* mutations suppressed the Fix^- phenotype of *phoCDET* mutants by allowing the *orfA-pit* system to be expressed (5, 6). We constructed *phoC pit* double mutants in RCR2011 (RmP633) and RmP110 backgrounds (RmP636), in which the *pstSCAB* genes were wild type, and both the double mutants formed normal wild-type N_2-fixing root nodules (Table 3). Thus, the results of this study, together with previous reports, show that expression of a single functional P_i transport system, be it OrfA-Pit, PstSCAB, or PhoCDET, is necessary and sufficient for symbiotic N_2 fixation in alfalfa.

It is important to consider the data and conclusions reported here, together with previously published microarray data (36) regarding the pleiotropic effect of the *pstC1021* mutation in Rm1021, when genomic experiments with strain Rm1021 are considered. Thus, the partial Pho constitutive phenotype resulting from the *pstC1021* mutation appears to be responsible for the reported expression of *phoCDET* in microarray studies and the presence of *phoD* in proteomic studies of alfalfa bacteroids formed by Rm1021 (7, 22, 36). On the basis of nodule plant gene expression data and metabolite analysis of *Lotus japonicus* root nodules, Colebatch et al. (19) recently suggested that root nodules experience P limitation and that this could result from preferred acquisition of available P by bacteroids. Analysis of P metabolism in *Rhizobium tropici* and bean nodules suggested that the bacteroids are P_i limited (2). The expression of *orfA-pit* and not *pstSCAB* or *phoCDET* in RCR2011 bacteroids (Fig. 5 and 6) clearly suggests that metabolism in alfalfa bacteroids is not limited by P_i under the plant growth conditions employed in our experiments. However, it is possible that bacteroid metabolism and the high density of bacteroids in nodules are a considerable P_i sink and could lead to P stress conditions in the alfalfa plant cytoplasm. It would be interesting to determine whether manipulation of the amount of P_i available to the plant (38, 66) can alter the pattern of expression of the P_i transporters in bacteroids within the alfalfa nodule.

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